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Multiple Mutations Lead to MexXY-OprM-Dependent Aminoglycoside Resistance in Clinical Strains of *Pseudomonas aeruginosa*

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Constitutive overproduction of the pump MexXY-OprM is recognized as a major cause of resistance to aminoglycosides, fluoroquinolones, and zwitterionic cephalosporins in *Pseudomonas aeruginosa*. In this study, 57 clonally unrelated strains recovered from non-cystic fibrosis patients were analyzed to characterize the mutations resulting in upregulation of the *mexXY* operon. Forty-four (77.2%) of the strains, classified as *agrZ* mutants were found to harbor mutations inactivating the local repressor gene (*mexZ*) of the *mexXY* operon ($n = 33$; 57.9%) or introducing amino acid substitutions in its product, MexZ ($n = 11$; 19.3%). These sequence variations, which mapped in the dimerization domain, the DNA binding domain, or the rest of the MexZ structure, mostly affected amino acid positions conserved in TetR-like regulators. The 13 remaining MexXY-OprM strains (22.8%) contained intact *mexZ* genes encoding wild-type MexZ proteins. Eight (14.0%) of these isolates, classified as *agrW1* mutants, overexpressed the gene PA5471, which codes for the MexZ antirepressor AmrZ, with 5 strains exhibiting growth defects at 37°C and 44°C, consistent with mutations impairing ribosome activity. Interestingly, one *agrW1* mutant appeared to harbor a 7-bp deletion in the coding sequence of the leader peptide, PA5471.1, involved in ribosome-dependent, translational attenuation of PA5471 expression. Finally, DNA sequencing and complementation experiments revealed that 5 (8.8%) strains, classified as *agrW2* mutants, harbored single amino acid variations in the sensor histidine kinase of ParRS, a two-component system known to positively control *mexXY* expression. Collectively, these results demonstrate that clinical strains of *P. aeruginosa* exploit different regulatory circuitries to mutationally overproduce the MexXY-OprM pump and become multidrug resistant, which accounts for the high prevalence of MexXY-OprM mutants in the clinical setting.

Pseudomonas aeruginosa is a frequent cause of nosocomial infections and is associated with progressive lung deterioration in cystic fibrosis (CF) patients. In addition to its elevated intrinsic resistance to many anti-Gram-negative antibiotics, this pathogen is notoriously known for its ability to develop clinically relevant levels of resistance to the most potent antipseudomonal drugs available, including aminoglycosides (e.g., gentamicin, tobramycin, amikacin) (1). Besides the acquisition of plasmid- and/or integron-borne genes encoding various aminoglycoside-modifying enzymes, the major mechanism by which *P. aeruginosa* may readily decrease its susceptibility to these agents consists of production of an RND (resistance nodulation cell division family) efflux pump, MexXY-OprM (1). This multispecific active efflux system accommodates a large range of antimicrobials, including zwitterionic cephalosporins (cefepime, cefpirome), macrolides (e.g., erythromycin), fluoroquinolones (e.g., ciprofloxacin), and tetracyclines (e.g., tetracycline, tigecycline), in addition to aminoglycosides (2–9). Ribosome protection experiments have demonstrated that MexXY-OprM contributes to the natural resistance of *P. aeruginosa* to only those exported substrates able to induce *mexXY* operon expression, as a result of protein synthesis impairment (10). Of note, the outer membrane protein OprM, which interacts with the periplasmic adaptor MexX and the RND transporter MexY to form a functional tripartite efflux machinery (2), is encoded by the constitutively expressed operon *mexAB-oprM* (11, 12). This drug-dependent activation of *mexXY* was found to depend upon the expression of a gene of unknown function, PA5471, itself transcriptionally coupled with a nonessential gene (PA5470) encoding a putative accessory peptide-releasing factor (13). When *P. aeruginosa* is exposed to subinhibitory concentrations of ribosome-targeting drugs, such as aminoglycosides, tetracyclines, macrolides, and chloramphenicol (a poor if at all a substrate of the pump MexXY-OprM), the PA5471-70 operon is overexpressed (13). This induction process has been proposed to rely on a sophisticated transcription attenuation mechanism that involves a short leader peptide, PA5471.1, whose coding sequence is located 5′ upstream of the PA5471-70 transcript (14). In the absence of drug exposure, PA5471.1 was predicted to form a stem-loop structure with adjacent sequences on the leader mRNA ahead of PA5471-70, which coexists with a terminator-like second stem-loop thought to attenuate PA5471-70 transcription by RNA polymerase (14). Antibiotic interference with the translation process, possibly up to a point where ribosomes stall, would prevent the formation of these secondary mRNA structures and thus allow PA5471 gene expression and subsequent *mexXY* activation (14). The PA5471 product, recently renamed ArmZ (for antirepressor MexZ), was found to physically interact with and to negatively modulate the activity of MexZ, the local repressor of the operon *mexXY* (15, 16). The MexZ protein, which binds as a dimer to the divergent overlapping promoters of *mexZ* and *mexXY* in the *mexZ-mexXY* intergenic region, would be relieved from its DNA binding site through

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotype[a] or genotype	Reference or origin
P. aeruginosa strains		
PAO1	Wild-type reference strain PAO1 with resequenced genome	24
PAOW1	PAO1 spontaneous mutant overexpressing MexXY	24
PAOW2	PAO1 spontaneous mutant overexpressing MexXY with M59I substitution in response regulator ParR	24
CMZ091	PAO1 Δ*mexZ*	24
CM096	PAO1 Δ*parRS*	24
CM106	CM096 *cis* complemented with *parRS* from PAOW2, Tc^r	24
CM114	PAO1 Δ*mexXY*	This study
CM115	PAO1 Δ*oprM*	This study
CM116	CM096 *cis* complemented with *parRS* from PAO1, Tc^r	This study
CM141	CM096 *cis* complemented with *parRS* from strain 2756, Tc^r	This study
CM142	CM096 *cis* complemented with *parRS* from strain 2794, Tc^r	This study
CM143	CM096 *cis* complemented with *parRS* from strain 2946, Tc^r	This study
CM144	CM096 *cis* complemented with *parRS* from strain 3136, Tc^r	This study
CM145	CM096 *cis* complemented with *parRS* from strain 3562, Tc^r	This study
CM146	CM096 *cis* complemented with *parRS* from strain 3726, Tc^r	This study
CM147	CM096 *cis* complemented with *parRS* from strain 3728, Tc^r	This study
E. coli strains		
CC118	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	54
HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 pro A2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*	Invitrogen
Plasmids		
PCR2.1	Cloning vector for PCR products, *lacZ*ΔColE1 f1 *ori*, Ap^r Km^r	Invitrogen
pRK2013	Helper plasmid, ColE1 *ori*, Tra^+ Mob^+ Km^r	30
mini-CTX1	Self-proficient integration vector, *tet* Ω-*FRT-attP*-MCS *ori int oriT*, Tc^r	36

[a] Antibiotic selection markers: Tc^r, tetracycline; Ap^r, ampicillin; Km^r, kanamycin. MCS, multiple cloning site.

its interaction with ArmZ, thus resulting in hyperexpression of *mexXY* (15). Although this two-step regulatory pathway is activated when *P. aeruginosa* is challenged with protein synthesis inhibitors, some of which are poor MexXY-OprM substrates, no evidence has been obtained to indicate that these inducing agents directly interact with MexZ independently of ArmZ (i.e., with basal, uninduced PA5471 gene expression) (17). The constitutive overproduction of the proteins MexXY is generally associated with a 2- to 16-fold increase in the MICs of pump substrates (18, 19). In addition to CF and non-CF clinical MexXY-OprM-overproducing strains found to harbor mutations inactivating the gene *mexZ* (e.g., indels, premature stop codons) or its product, MexZ (e.g., amino acid substitutions), isolates harboring intact (wild-type) *mexZ* genes have repeatedly been reported (19–25) but have rarely been characterized (26). The observation that MexXY-OprM-upregulated mutants with defective protein synthesis can be selected *in vitro* on aminoglycoside (7, 26, 27) or a peptide deformylase inhibitor (28) suggested that such mutants, called *agrW1*, to differentiate them from *mexZ* mutants (dubbed *agrZ*), might be hypovirulent and thus poorly relevant in the clinic setting because of impaired fitness. Recently, our group reported that single amino acid changes in the response regulator ParR or the sensor kinase ParS of the two-component regulatory system ParRS may result in overexpression of both the *mexXY* operon and the lipopolysaccharide modification locus *arnBCADTEF-ugd*, along with decreased transcription of the gene for the porin OprD (24). Mutants (called *agrW2*) harboring alterations in ParRS have been identified in the clinical setting (24, 29), some of which were from severely ill patients admitted to intensive care units (ICU). The objective of the present study was to evaluate the prevalence of and to better characterize the three known types of MexXY-OprM-overexpressing strains (i.e., *agrZ*, *agrW1*, and *agrW2*) in a collection of genotypically characterized clinical strains of *P. aeruginosa* resistant to aminoglycosides.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. In addition, 92 nonconsecutive clinical *P. aeruginosa* isolates were selected from our laboratory collection. These strains, which were collected between 2001 and 2009 at the University Hospital of Besançon (France), were chosen for their typical MexXY-OprM-dependent resistance profile, characterized by an at least 2-fold increase in the MIC of three or more of the following substrates compared with wild-type strain PAO1: tobramycin (≥1 μg ml^{-1}), amikacin (≥8 μg ml^{-1}), apramycin (≥32 μg ml^{-1}), ciprofloxacin (≥0.5 μg ml^{-1}), and/or cefepime (≥8 μg ml^{-1}). Multidrug-resistant bacteria exhibiting elevated resistance to amikacin (≥128 μg ml^{-1}), apramycin (≥512 μg ml^{-1}) and/or cefepime (≥64 μg ml^{-1}) were not retained because of probable expression of enzymatic mechanisms. All the bacterial cultures were grown in Mueller-Hinton broth (MHB) with adjusted concentrations of Ca^{2+} (from 20 to 25 mg/liter) and Mg^{2+} (from 10 to 12.5 mg/liter) (Becton, Dickinson and Company, Cockeysville, MD), or on Mueller-Hinton agar (MHA; Bio-Rad, Marnes la Coquette, France). Recombinant plasmids were introduced into *P. aeruginosa* strains by triparental matings and mobilization via the broad-host-range vector pRK2013 harbored by *Escherichia coli* HB101 (30). Transconjugants were selected on pseudomonas isolation agar (PIA; Becton, Dickinson and Company) supplemented with 150 μg ml^{-1} ticarcillin, 200 μg ml^{-1} tetracycline, or 2,000 μg ml^{-1} streptomycin, as required. Transformants of *E. coli* were selected on MHA containing 100 μg ml^{-1} ampicillin, 15 μg ml^{-1} tetracycline, or 50 μg ml^{-1} streptomycin. Growth curves of selected *P. aeruginosa* strains were established in triplicates by monitoring the absorbance at 600 nm of bacteria developing aerobically in 30 ml MHB at 37°C or 44°C under vigorous shaking (250 rpm).

Antibiotic susceptibility testing. The MICs of selected antibiotics were determined by the standard serial 2-fold dilution method in MHA with an inoculum of 10^4 CFU per spot, as recommended (31). Colistin MICs were determined by the macrodilution MHB procedure (31). Growth was assessed visually after 18 h of incubation at 35°C to 37°C.

Mapping of *mexZ* mutations. The three-dimensional (3D) structure of MexZ (32) is available in the protein data bank (PDB; 2WU1; http://www.rcsb.org/pdb/home/home.do). Its structure has been solved in the H32 space group with one monomer per unit cell. We generated the functional dimer by application of a crystallographic symmetry to analyze the consequences of the different mutations at the interface. In addition, to be able to analyze the DNA binding surface, a model was built of MexZ in complex with DNA. For that purpose, a search for DNA-bound TetR family regulators was performed with the PDB database, resulting in 4 different structures. The one presenting the smallest RMSD (root mean square deviation) with the MexZ monomer was further used as a template. It was the TetR/DNA structure complex (PDB code 1QPI) (33). The MexZ/DNA model was built by direct superposition of the two MexZ monomers on the two TetR ones by using the coot program (34). With 29% of sequence homology between the two proteins, the superposition led to an RMSD of 12 Å, calculated on the 2 times 135 common Cα atoms. Mutations found in clinical strains were visualized on both the free and DNA-bound models of MexZ by using MacPyMol software (DeLano Scientific, LLC).

Alignment of the protein MexZ with various TetR-like homologs. Multiple sequence alignment of the MexZ protein from *P. aeruginosa* was performed with homologs from different species by using the ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) in order to identify conserved regions. The different sequences were retrieved from the UniProt database (http://www.uniprot.org/uniprot/), and Jalview software was used to edit the alignments (35).

Chromosomic complementation with full-length *parRS*. The *parRS* loci of strains exhibiting mutations in the gene *parS* were amplified by PCR from genomic DNA using the primers CloparRSC1/CloparRSC2 or CloparRSBamHIS1/CloparRSHindIIIS2 (Table 2). The resulting DNA fragments were cloned either in pCR2.1 and next ligated to the EcoRV/SpeI restriction sites of plasmid mini-CTX1 or were cloned directly in BamHI/HindIII-restricted mini-CTX1 (36). The recombinant plasmids were then transferred from *E. coli* CC118 to *P. aeruginosa* strain CM096 (PAO1Δ*parRS*) by conjugation with subsequent selection on tetracycline-PIA plates, to allow their chromosomal insertion into the *attB* site.

Gene transcript quantification by RT-qPCR. Specific gene expression levels were measured by real-time, quantitative PCR (RT-qPCR) after reverse transcription, as described previously (37). Briefly, 2 μg of total RNA was reverse transcribed with ImpromII reverse transcriptase as specified by Promega (Madison, WI). The amounts of specific cDNA were assessed in a Rotor Gene RG6000 real-time PCR instrument (Qiagen, Courtaboeuf, France) by using the QuantiFast SYBR PCR green kit (Qiagen), primers designed from the sequence in the *Pseudomonas* Genome Database, version 2 (Table 2), and *rpsL* transcripts as internal controls. The mRNA levels of target genes were normalized for each strain with those of *rpsL* and expressed as a ratio (the fold change) compared to that of wild-type strain PAO1, used as the reference. Mean gene expression values were calculated from two independent bacterial cultures, each assayed in duplicate. As established previously, transcript levels of *mexY* that were ≥4-fold above that of strain PAO1 were considered significantly increased in clinical strains (9). Well-characterized MexXY-overproducing mutants derived from PAO1 (CMZ091, PAOW1, and PAOW2) were used as positive controls to assess the overexpression of genes *mexY*, PA1797, PA5471, and *arnA* (24).

Genotype analysis. The clonal relatedness of isolates was investigated by using Clondiag chips (Alere, Sèvres, France), which allows the identification of 14 single nucleotide polymorphisms (SNPs) in the core genome of *P. aeruginosa*. A genetic fingerprint (referred to as the hexadecimal code) was assigned to each strain and corresponded to the SNP combination found (38).

TABLE 2 Nucleotide primers

Purpose or target and primer name	Nucleotide sequence[a] (5′ → 3′)
Gene sequencing	
mexZ and *mexX-mexZ* intergenic region	
SeqZ1	GCAGCCCAGCAGGAATAG
SeqZ2	GCCTGTCGGTGCTCTACATC
parRS	
SeqparSC1	GCCAGGCAGGGGAAATACT
SeqparSC2	CATACCAGCAGGGCGGATG
SeqparSC3	AAGAACCTGCTGGTGGTACG
SeqparSC4	ATGCGGATCTGTTCGACCT
SeqparSC5	CGAACTGGAGGAAATGGTCT
SeqparSC6	GAAAGATGCATTGCACGAAA
SeqparRC1	GCATATAATGCCAGCCGATT
SeqparRC2	GGTCGACCACGAAGATCG
PA5471.1	
SeqPA5471.1S1	GGAGTCCTTCATGACCTTCG
SeqPA5471.1S2	GTGGTTTGCAGTTGCTGGAT
Cloning of genes *parRS* for *cis* complementation	
CloparRSC1	GAGGGAAAAGCAGAAGTCACC
CloparRSC2	CGAGGTGTCCCATGCTAGG
CloparRSBamHIS1	CTCGGATCCGAGGGAAAAGCAGAAGTCACC
CloparRSHindIIIS2	CTCAAGCTTCGAGGTGTCCCATGCTAGG
Gene expression measurement by RT-qPCR	
RTrpsL1	GCAACTATCAACCAGCTGGTG
RTrpsL2	GCTGTGCTCTTGCAGGTTGTG
RTmexY1A	TTACCTCCTCCAGCGGC
RTmexY1B	GTGAGGCGGGCGTTGTG
RTPA5471C1	GAAGGCAAGGCGATCCAG
RTPA5471C2	AGGCGCTTTTCCAGTTTGT
RTPA1797C1	GGACCCTTTGCAGATGACTC
RTPA1797C2	CGGAGTGTTTCCTGAGAAGC
RTarnAC1	GTGGCTCGAATACCATGTGA
RTarnAC2	TGCCGTATTTCACGCAGTAG

[a] The recognition sequences of endonucleases BamHI and HindIII are underlined.

RESULTS AND DISCUSSION

Selection of clinical strains overproducing MexXY. In order to characterize the different types of *mexXY*-upregulated mutants occurring in the clinical setting, we screened a collection of 92 *P. aeruginosa* isolates that exhibited a reduced susceptibility to typical MexXY-OprM substrates, including aminoglycosides (tobramycin, amikacin, apramycin), ciprofloxacin, and/or cefepime. Subsequent RT-qPCR experiments confirmed that all the selected bacteria overexpressed the gene *mexY* significantly above the wild-type reference strain PAO1 expression level. As demonstrated by SNP analysis with Clondiag chips (Alere), 42 strains were unique, while 9, 1, 2, 1, 1, and 1 other were represented by 2, 3, 4, 5, 6, and 10 isolates, respectively. Incidentally, these genotyping experiments pointed to the spread and persistence for years of several MexXY-OpM-overproducing clones in the hospital (data not shown). Finally, 57 genotypically distinct strains were retained for further investigations. As reflected by the MIC_{50} values shown in Table 3, the median resistance levels to MexXY-OprM substrates were close to those of typical *in vitro*-selected *agrZ* (CMZ091), *agrW1* (PAOW1), and *agrW2* (PAOW2) mutants. Most of these bacteria were susceptible to tobramycin (91%) and amikacin (84%) according to the current CLSI breakpoints, and they were more resistant to cefepime than to ceftazidime, as reported previously (9). For apramycin, which is recalcitrant to many aminoglycoside-modifying enzymes, the MIC values were ≥32 μg ml^{-1} in 55 strains of the selection (96.5% positivity).

***mexZ*-deficient mutants.** Sequence analysis of *mexZ*, which encodes the local repressor of the operon *mexXY*, revealed that

TABLE 3 Drug resistance levels of the 57 selected clinical isolates

	Resistance levels of clinical isolates					Reference strain MICs (μg/ml)			
		% in resistance category[a]							
Antibiotic	MIC range (μg/ml)	S	I	R	MIC_{50}/MIC_{90}	PAO1	CMZ091	PAOW1	PAOW2
Tobramycin	0.5–128	91	0	9	1/4	0.5	1	2	1
Amikacin	4–64	84	14	2	16/32	4	8	16	8
Apramycin	16–256	ND	ND	ND	64/128	16	32	64	64
Ciprofloxacin	0.125–64	67	5	28	0.5/32	0.25	0.5	0.5	0.5
Cefepime	2–32	53	30	17	8/32	4	8	16	8
Ceftazidime	0.5–32	95	3	2	2/8	2	2	2	2
Ticarcillin	4 to >1,024	42	51	7	32/64	16	16	16	16

[a] According to the current CLSI breakpoints for tobramycin (S, ≤4 μg/ml; R, ≥16 μg/ml), amikacin (S, ≤16 μg/ml; R, ≥64 μg/ml), ciprofloxacin (S, ≤1 μg/ml; R, ≥4 μg/ml), cefepime (S, ≤8 μg/ml; R, ≥32 μg/ml), ceftazidime (S, ≤8 μg/ml; R, ≥32 μg/ml), ticarcillin (S, ≤16 μg/ml; R, ≥128 μg/ml). I, intermediate; ND, not defined.

33/57 (57.9%) strains carried a mutated, inactive gene due to frameshifts (29/33), premature stop codons (3/33), or codon deletions (1/33). In addition to these *agrZ* mutants, 11/57 (19.3%) strains appeared to harbor point mutations resulting in one (9 isolates) or two (2 isolates) amino acid substitutions in the repressor MexZ, while 13/57 (22.8%) contained *mexZ* genes encoding wild-type PAO1 or PA14 proteins (by definition, *agrW* mutants) (see Table S1 in the supplemental material).

To better understand how the 13 mutations found in MexZ impact its activity, we mapped these variations in our models of free- and DNA-bound MexZ structures (Fig. 1). The mutated residues could be classified into three different groups with respect to their location in the folded protein. Because they affect the dimer interface, the first group of mutations (Gly172Asp, Gly195Glu, and Ser198Ile) are predicted to strongly impair the dimerization of MexZ and its subsequent binding to the regulatory sequences upstream of *mexXY*. Of note, Gly195Glu was also identified in an extremely drug-resistant

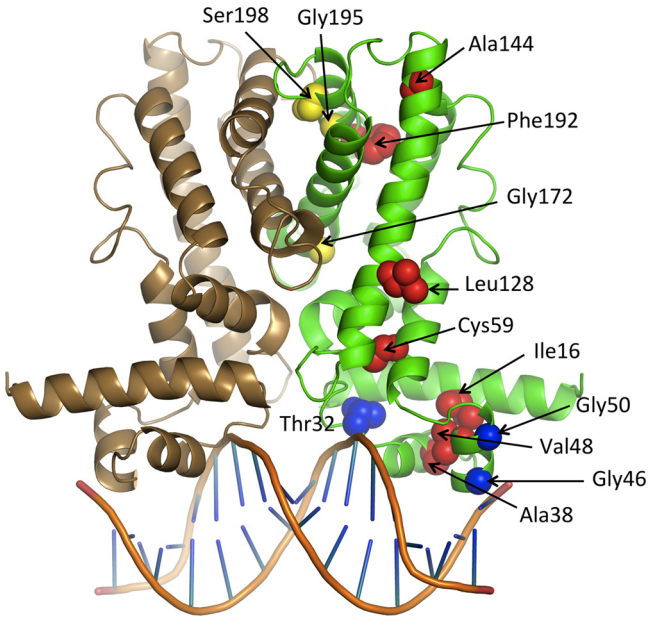

FIG 1 Three-dimensional illustration of MexZ mutations, localized on the MexZ/DNA model built for this study. The two molecules of the dimer are colored green and brown, respectively. Residues are represented by spheres in yellow for those located at the dimer interface, in blue for those located at the protein-DNA interface, and in red for all the other.

(XDR) international epidemic clone belonging to the same sequence type, ST175 (39), as strain 2508 and 4 clonally related isolates originally selected for this study (data not shown). In a future study, sequencing of *mexZ* genes from ST175 strains collected in various countries could confirm whether Gly195Glu is a signature for this successful clonal complex.

Mutations of the second group, which mapped in the N-terminal helix-turn-helix domain of MexZ (Thr32Asn, Gly46Ser, and Gly50Asp) are also expected to abrogate DNA binding. It should be noted that Thr32Asn was associated with group 1 mutation Ser198Ile in strain 4465. Finally, mutations of the third group were distributed all along the rest of the structure. All of them except Leu128Met are assumed to disturb the 3D structure of MexZ, because of their location in the core of the structure (Cys59Tyr, Phe192Tyr) or at helix interfaces (Ile16Thr, Ala38Val, Val48Ala, Ala144Val). Phe192Tyr was associated with group 1 mutation Gly172Asp in strain 3245. Val48Ala was previously reported in MexXY-overexpressing multidrug-resistant *P. aeruginosa* isolates from Germany (40). In addition to pure structural effects, some additional points should be noted. Phe192 takes part in the antibiotic binding pocket present in most members of the TetR family, even if it has been suggested that MexZ does not interact with antibiotics even though the cavity is present (32). Cys59 is at the bottom of the external helix (α4) that is supposed to transmit the protein conformational changes to its DNA binding domain (41). Its mutation into Tyr could rigidify the helix locally and abrogate this transmission. Ala38 is very close to the DNA fragment in the complex model. Consequently, it could also be considered a DNA binding actor, as structure modifications not taken into account in our model can occur when MexZ binds to DNA. Finally, the consequence of the last mutation, Leu128Met in strain 3135, is more difficult to explain, as it is located at the outer surface of the molecule. Nevertheless, in the structure of SimR (42), a TetR family transcriptional regulator from *Streptomyces antibioticus*, a two-helix insertion before the C-terminal helix and an N-terminal extension that covers the region containing the equivalent residue to Leu128 is supposed to play a regulatory role. Thus, it can be hypothesized that the Leu128Met change disturbs the regulatory function of MexZ, maybe via its interaction with another partner.

***agrW1*-type mutants.** As reported above, 13 MexXY-OprM overproducers exhibited a wild-type, intact repressor MexZ compared with PAO1 and PA14. RT-qPCR experiments were performed to characterize these strains further (Table 4). Strongly

TABLE 4 Genetic and phenotypic features of the eight *agrW1* and five *agrW2* mutant clinical strains

Strain	Genotype	Mutation in *parR*	Mutation(s) in *parS*	MIC[a] (μg/ml)								Expression level[b]			
				TOB	AMK	APR	CIP	FEP	CAZ	TIC	CST	*mexY*	PA5471	PA1797	*arnA*
PAO1	Wild type			0.5	4	16	0.25	4	2	16	1	1	1	1	1
PAOW1	*agrW1*			2	16	64	0.5	16	2	16	1	15.2	6.1	−1.2	1.0
PAOW2	*agrW2*	M59I		1	8	64	0.5	8	2	16	2	16.0	−1.6	92.9	104.7
2405	*agrW1*			4	64	128	0.5	8	2	32	1	39.5	6.0	1.2	−5.0
2946	*agrW1*		R155H[c]	1	16	32	1	16	2	64	1	35.9	4.8	6.3	4.6
3136	*agrW1*		A82T[c]	2	32	128	0.5	8	2	32	1	18.2	3.5	−1.2	−1.4
3452	*agrW1*			4	32	256	32	8	2	32	1	16.2	10.1	3.3	−2.2
3564	*agrW1*			4	32	256	0.5	8	1	16	1	43.9	5.0	−1.2	−1.4
4192	*agrW1*			1	8	32	0.5	8	2	32	1	44.7	15.6	−1.2	−1.6
4592	*agrW1*			2	16	64	4	16	16	64	1	16.9	4.6	1.1	2.1
5013	*agrW1*			1	16	64	1	16	4	32	1	51.3	7.4	4.0	−1.4
2756	*agrW2*		A168V[d]	1	16	32	2	16	4	64	1	8	−1.1	12.0	96.3
2794	*agrW2*		L99P[d]	4	16	128	2	32	4	32	2	28.7	1.3	40.7	17.6
3562	*agrW2*		L137P[d]	128[e]	32	64	0.5	16	4	>1,024[e]	4	8.0	−1.1	3.0	98.7
3726	*agrW2*		V152A[d]	2	16	64	0.5	8	2	16	1	10.5	1.4	27.9	28.7
3728	*agrW2*		R83K, A138T[d]	2	16	64	1	8	2	16	2	4.3	1.2	4.6	11.0

[a] Abbreviations: TOB, tobramycin; AMK, amikacin; APR, apramycin; CIP, ciprofloxacin; FEP, cefepime; CAZ, ceftazidime; TIC, ticarcillin; CST, colistin.
[b] Mean values of two independent experiments, expressed as ratios versus PAO1 expression levels.
[c] Amino acid change with no effect on protein function, as demonstrated by gene *cis* complementation of mutant strain PAO1Δ*parRS*, CM096 (see Table S1 in the supplemental material).
[d] Amino acid change activating sensor ParS (see Table S1).
[e] Probable additional enzymatic mechanism of resistance.

supporting the notion that 8 strains could be classified as *agrW1*-type mutants, expression levels of the gene PA5471, which codes for the antirepressor ArmZ (16), turned out to be 3.5- to 15.6-fold higher in these bacteria than in PAO1 (levels were increased 6.1-fold in the *in vitro*-selected mutant PAOW1 [Table 4]). We thus looked for mutations in the loci already known to affect ribosomal functioning and to trigger constitutive, drug-independent activation of the operon *mexXY* through the PA5471 gene product. However, analysis of the coding sequences of genes *rplA* (7), *rplY* (27), *fmt* (28), and *folD* (28), as well as of the promoter region of the *rplU-rpmA* operon (26), failed to reveal significant alterations (i.e., nonsynonymous mutations, nucleotide insertions or deletions, or premature stop codons) compared with strains PAO1 and PA14. This result was not surprising *per se*, as multiple drug-dependent and drug-independent events are known to activate PA5471 expression as far as the translational machinery is perturbed (43). Since the *mexXY* operon is activated via a transcription attenuation mechanism involving the nucleotide sequence of the leader peptide PA5471.1 when ribosomes are stalling (14), we made the assumption that some ribosome-perturbing mutations might affect protein synthesis and growth rates. Indeed, alteration of *rplA*, *rplY*, *fmt*, *folD*, or the *rplU-rpmA* promoter region negatively impacts the fitness of *P. aeruginosa* (7, 26, 27, 44). Consistent with our hypothesis, 5 of the 8 clinical strains (2405, 2946, 3136, 3564, and 5013) exhibited a longer lag phase, slower growth rates, and/or earlier onset of stationary phase than PAO1 at 37°C (Fig. 2A). The deficient fitness of these strains was much more evident when the incubation was performed at 44°C (Fig. 2B), a condition which almost inhibited their growth. The characterization of ribosomal mutations responsible for MexXY derepression in strains 3136, 3564, and PAOW1 is currently in progress (unpublished data). Contrasting with these findings, the 3 remaining strains, 3452, 4192, and 4592, multiplied like PAO1 at both temperatures (Fig. 2A and B), suggesting that all the PA5471-inducing mutations are not necessarily associated with a significant fitness cost for *P. aeruginosa*.

A deletion in PA5471.1 leads to upregulation of PA5471. Sequencing of the 367-bp intergenic region between PA5472 and PA5471 did not reveal mutations in the *agrW1* strains except in 4192, where the 42-bp coding sequence of the leader peptide gene PA5471.1 was found to be disrupted by a 7-bp deletion (Δ16–22). In agreement with this, Morita et al. demonstrated by *in vitro* site-directed mutagenesis that interference with gene PA5471.1 translation results in PA5471 gene overexpression in strain PAO1 (14). In the absence of mutations (e.g., generating premature stop codons), the wild-type PA5471.1 transcript was predicted to be involved in a stem-loop structure (named PA5471.1-2) on the nascent mRNA ahead of gene PA5471, which coexists with a second stem-loop (PA5471.1 3-4), with characteristics of putative transcription terminators. This presumed structure would prevent the transcription of the PA5471-70 operon (14). Because the 7-bp deletion found in strain 4192 would preclude the formation of the proposed PA5471.1-2 structure, other mRNA base-pairing rearrangements might lead to the disruption of the uracyl-rich transcription terminator and promote high-level constitutive expression of genes PA5471-70 (14). If confirmed, the mechanism found in strain 4192 would artificially reproduce some of the conformational changes occurring in the leader region of PA5471 mRNA when the transcriptional and the translational rates are perturbed (uncoupled) by antibiotics.

***agrW2*-type mutants.** The 5 remaining strains (2756, 2794, 3562, 3726, and 3728) showed increased transcript levels of genes PA1797 and *arnA* due to alteration of the sensor kinase ParS from the two-component system ParRS (24). Confirming recent data on imipenem-resistant ICU strains (29), most of the mutations mapped in the periplasmic domain (R83K, L99P) or in the second transmembrane segment (L137P, V152A, A138T) of ParS, which is known to be important for signal transduction. The A168V

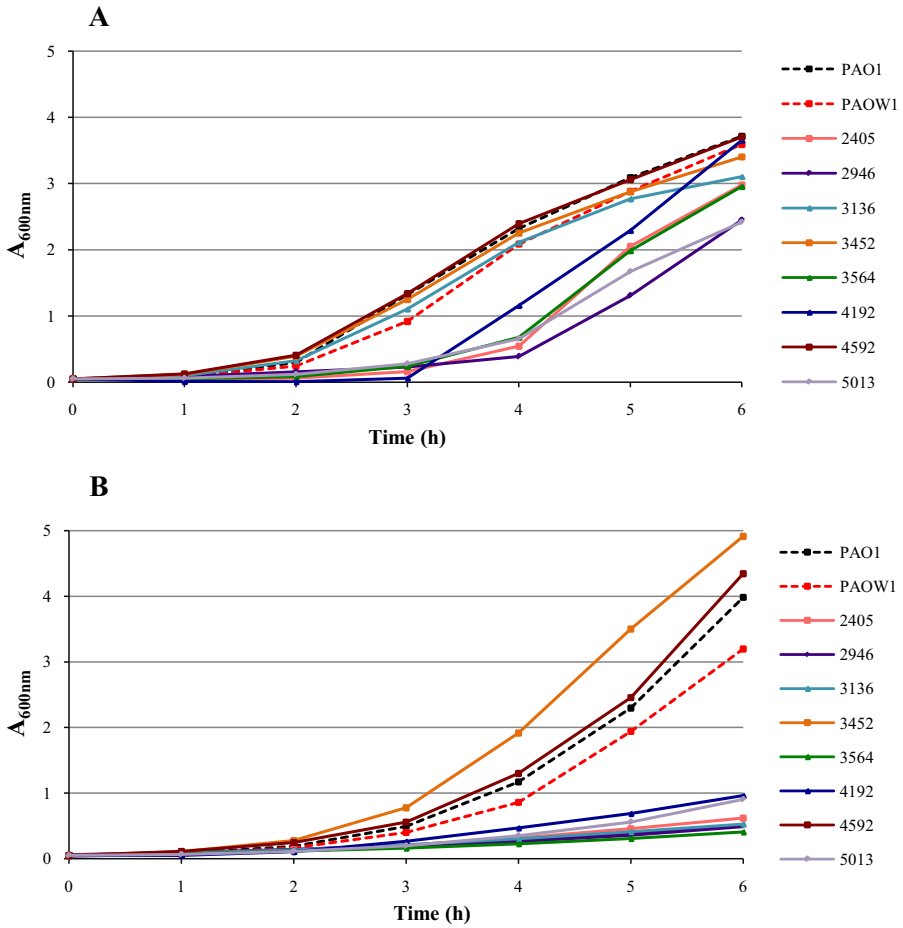

FIG 2 Representative growth curves of *agrW1* mutants at 37°C (A) and 44°C (B). Bacteria were cultured in triplicates in MHB with an initial inoculum of 0.1 at A_{600}. Because of the formation of bacterial aggregates, growth of strain 4192 could not be reliably monitored by spectrophotometry.

change harbored by strain 2756 was located in the HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis proteins and phosphatases) domain, which physically connects the signal-sensing/transducing input functions of periplasmic and transmembrane regions to the kinase output functions of the cytoplasmic domain (45). It should be noted that mutations at positions L137 (24) and A138 (29) have already been associated with ParRS-dependent *mexXY* overexpression in clinical strains. Of note, A138T coexisted with R83K in strain 3728, indicating that several mutations may accumulate in the *parS* gene. To confirm the role played by the aforementioned mutations in *mexXY* operon upregulation, the mutated *parRS* loci were cloned and inserted as single copies in the *attB* site of PAO1Δ*parRS* (mutant CM096) chromosome. As expected, the mutated *parRS* increased the resistance of CM096 to some or all of the MexXY-OprM substrates tested, as well as to imipenem, a result consistent with the observation that, when activated, the two-component system ParRS negatively regulates porin OprD gene expression and thus promotes carbapenem resistance (24, 29) (see Table S2 in the supplemental material). In contrast, the ParS sequence polymorphism found in strains 2946 (R155H) and 3136 (A82T) did not influence the CM096 resistance levels significantly, suggesting no or faint activation of ParS in these strains, although the mutations lie in the HAMP and the periplasmic sensor domains, respectively, and despite a modest overexpression of genes PA1797 and *arn* in strain 2946 (Table 4; see also Table S2). Finally, as noted previously (24), mutational activation of ParRS was accompanied in some clinical *agrW2* strains by a marginal increase in the colistin MIC (from 2- to 4-fold above that for PAO1 [Table 4]).

Conclusions. The present study shows that a variety of specific and aspecific mutations affecting three distinct regulatory pathways actually lead to the overexpression of efflux system MexXY and to increased aminoglycoside resistance in clinical strains of *P. aeruginosa*. These pathways involve the local repressor MexZ (*agrZ*-type mutants), the MexZ antirepressor ArmZ (*agrW1*-type mutants), and the two-component regulatory system ParRS (*agrW2*-type mutants). Consistent with previous data (8, 19, 20, 22, 23, 39, 46–51), we found that most of the clinical MexXY-overproducing strains harbored mutations that inactivated the gene *mexZ* or compromised the activity of its product through amino acid substitutions concentrating in positions that are conserved in TetR regulators, including the DNA binding and the dimerization domains (see Fig. S1 in the supplemental material). It should be noted that mutational activation of the PA5471-dependent or/and the ParRS-dependent regulatory pathways was not systematically investigated in the *mexZ*-deficient strains, which may have led to an underestimation of potential double or triple *agrZ agrW1 agrW2* mutants in the clinical setting. *In vitro*

inactivation of the *mexZ* gene in laboratory mutants PAOW1 (data not shown) and PAOW2 (24) of the *agrW1* and *agrW2* types, respectively, could not demonstrate additive effects of the different MexXY-activating mutations on aminoglycoside resistance levels. Therefore, though the occurrence of such double or triple mutants cannot be ruled out in patients, their therapeutic relevance is expected to be the same as those of single mutants. We and others (21, 22) failed to identify mutations in the *mexZ-mexXY* intergenic regions of MexXY overproducers, which is somewhat intriguing. Since the expression of *mexZ* and *mexXY* is governed by overlapping promoter sequences encompassing the 20-bp palindromic binding site of repressor MexZ (15), a plausible explanation would be that mutations downregulating *mexZ* also negatively influence *mexXY* expression. Consistent with this, we found that a 3-bp deletion in the region where repressor MexR binds to the *merR* and *mexA* overlapping promoters downregulates the expression of operon *mexAB-oprM* and decreases antibiotic resistance in clinical *P. aeruginosa* (52).

More surprising was the observation that *agrW1* mutants may survive in the clinical setting while being deficient in protein synthesis. With one exception (26), such mutants have not been reported previously to infect or colonize patients. Unfortunately, the clinical data associated with our collection were not sufficient to determine if some *agrW1* mutants remained pathogenic (i.e., were responsible for infections and not just colonization). One can imagine that those with unaffected growth rates may retain the ability to multiply actively in the immunocompromised host. Although there is increasing evidence that multiple ribosome-targeting mutations may result in PA5471-dependent upregulation of *mexXY* (7, 26–28), it is clear from this study that mutational disruption of the leader peptide PA5471.1, encoded by the untranslated region (5′ UTR) upstream of the gene PA5471, can also be exploited by *P. aeruginosa* to develop multidrug resistance. Other examples of mutation-driven shortcuts in ribosome-mediated attenuation of resistance genes have been reported for other organims [e.g., (*erm*C)] (53). Whether the 7-bp deletion characterized in strain 4192 prevents the formation of a terminator-like stem-loop ahead of operon *mexXY* or forces the ribosome to pause, allowing *mexXY* transcription, is unclear at the moment. Finally, this work confirmed the clinical relevance of *agrW2* mutants, which combine an efflux-based resistance to aminoglycosides, cefepime, and ciprofloxacin with a decreased outer membrane permeability to carbapenems (24). In agreement with these findings, similar mutants were recently isolated from patients in intensive care units (29). Further investigations will determine if, in addition to the very prevalent *agrZ* mutants (48, 49), *agrW1* and *agrW2* strains also occur in cystic fibrosis patients and enable *P. aeruginosa* to adapt to iterative aminoglycoside treatments in a context of chronic infection.

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